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Award Number: DAMD17-03-1-0487

TITLE: Fusions of Breast Carcinoma and Dendritic Cells as a Vaccine for the Treatment of Metastatic Breast Cancer

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REPORT DATE: July 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 1-07-2007 | Final | 1 JUL 2003 - 30 JUN 2007 |

**4. TITLE AND SUBTITLE**

Fusions of Breast Carcinoma and Dendritic Cells as a Vaccine for the Treatment of Metastatic Breast Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
DAMD17-03-1-0487

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Dana-Farber Cancer Institute
Boston, MA 02115

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
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**15. SUBJECT TERMS**
Breast cancer cells, dendritic cells, fusions, vaccines, T cells

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 13 | **19b. TELEPHONE NUMBER** *(include area code)* |

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**Vaccination of Patients with Metastatic Breast Cancer with Dendritic Cell/Breast Cancer Fusions in Conjunction with IL-12**
**Department of Defense Grant # DAMD17-03-1-0487**
**Progress Report Year 4 July 2007**

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**Baldev Vasir, PhD**
**Zekui Wu, MD**
**Donald Kufe, MD**

## Introduction

The overall objective of the project is to study the safety, immunologic response, and clinical effect of vaccination with dendritic cell (DC)/breast cancer fusions administered in conjunction with IL-12 in patients with metastatic breast cancer. DC/breast carcinoma fusion cells present a broad array of tumor-associated antigens in the context of DC-mediated costimulation. Fusion cells stimulate tumor specific immunity with the capacity to lyse autologous tumor cells. In clinical studies, vaccination with fusion cells was well tolerated, induced immunologic responses in a majority of patients, and results in disease regression in subset of patients. We postulated that administration of the vaccine in conjunction with IL-12 would further enhance vaccine response by promoting T cell activation.

In the first 3 years of the grant, we examined DC/breast carcinoma fusions with respect to their phenotypic characteristics as antigen presenting cells and their capacity to stimulate anti-tumor immunity. We demonstrated that DC/breast carcinoma fusions strongly express costimulatory, adhesion, and maturation markers as well as the stimulatory cytokines, IL-12 and IFNγ. In addition, fusion cells expressed CCR7 necessary for the migration of cells to sites of T cell traffic in the draining lymph nodes. In concert with these findings, fusions generated with immature and mature DCs potently stimulated CTL mediated lysis of autologous tumor targets.

We subsequently examined the T cell response to DC/breast carcinoma fusions with respect to the presence of activated and regulatory T cells. We demonstrated that DC/breast carcinoma fusions stimulate a mixed population of cells consisting of CD4/CD25/CD69 and CD4/CD25/Foxp3+ cells. The increased presence of regulatory cells was thought to potentially inhibit the in vivo efficacy of the fusion cell vaccine. As such, we have examined several strategies to bias the fusion-mediated T cell response towards activated cells. We have found that addition of IL-12, TLR7/8 agonists, CPG ODN, or IL-18 increased the relative presence of activated as compared to regulatory cells. Importantly, we have also found that DC/breast carcinoma fusion-induced activation of autologous T cells and then stimulation with anti-CD3/CD28 results in a marked expansion of anti-tumor effector cells.

## Body

### Induction of regulatory $CD4^{+}CD25^{+high}$ T cells in DC/tumor fusion cocultures

We have previously demonstrated that DC/breast carcinoma fusions stimulate the expansion of T cell populations that manifest phenotypic characteristics of regulatory and activated T cells. To further define the T cell response to DC/breast carcinoma fusions, we have examined the functional characteristics of the expanded T cell population that co-express CD4 and CD25. Following stimulation with fusion cells, increased induction of $CD4^{+}CD25^{+high}$ with high mean fluorescence intensity are observed as shown in Fig. 1A. To confirm the regulatory phenotype of this population of $CD4^{+}CD25^{+high}$ cells, their ability to suppress the activation of $CD4^{+}CD25^{-}$ cells was assessed using a co-culture in vitro proliferation assay.

Using strict gating parameters reported recently (1), regulatory T cells were identified as CD4 positive cells with a brighter CD25 staining than that of the CD4-negative or CD4 intermediate population (Fig. 1A). The frequency of regulatory T cells was calculated as the percentage of $CD4^{+}CD25^{+high}$ cells in the CD4+ population, and was in the range of 0.09-3% of total CD4+ T cells analyzed. The $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+high}$ population of cells were isolated from an average of 40-50 $x10^{6}$ positively selected CD4+ T cells from the DC/breast tumor fusion cell coculture (>97 % purity) using a FACSVantage SE (BD Biosciences, San Jose, CA). These cells were incubated with 100 µl each of anti-CD4 TC (IgG2a, Invitrogen) and anti-CD25-FITC (IgG1, BD PharMingen). The analysis and sort gates were restricted to the viable population of CD4+ T cells by means of their forward and side scatter properties. Large, activated CD4+ T cells were excluded as described (1,2). On reanalysis, the forward and side scatter properties of the $CD4^{+}CD25^{+high}$ cells were not appreciably different from those of the $CD4^{+}CD25^{-}$ population, suggesting that these cell populations are similar in size. Bidimensional flow cytometric analysis of CD4+ T cells positively selected from the DC/breast carcinoma fusion cell cocultures demonstrated the highest level of CD25 ($CD4^{+}CD25^{+high}$ with high mean fluorescence intensity) appearing as a tail to the right from the major population containing both $CD4^{+}CD25^{+low}$ and $CD4^{+}CD25^{-}$ cells as compared to unstimulated T cells (Fig. 1A). The $CD4^{+}CD25^{+high}$ represents between 1-3% of the total $CD4^{+}$ T cell population, whereas the $CD4^{+}CD25^{+low}$ cells represent up to 16% of CD4+ T cells as reported by others (1, 2, 3).

For suppression assays, $CD4^{+}CD25^{-}$ T cells ($5x10^{4}$cells/well) were cocultured in triplicate in the presence or absence of $CD4^{+}D25^{+high}$ T cells ($5x10^{4}$cells/well, 1:1 ratio) with Tetanus Toxoid (10 µg/ml) or with 1 µg/ml of anti-CD3 antibody (clone UCHT1; BD PharMingen) in the presence of irradiated (3500 Rad) T-cell depleted autologous PBMNCs ($2.5x10^{5}$ cells/well) as antigen presenting cells (APCs). As a control, equal numbers of $CD4^{+}CD25^{-}$ cells were added. APCs were isolated by negative selection of autologous PBMNCs incubated with anti-CD3 coated

magnetic beads (Miltenyi Biotec, CA). In addition, $CD4^+CD25^-$ T cells were stimulated with PHA (4 µg/ml) in the presence or absence of $CD4^+D25^{+high}$ T cells. The ability of $CD4^+CD25^{+high}$ T cells to suppress proliferation was assessed by [$^3$H]Thymidine (1 µCi [0.037 MBq] per well) incoporation pulsed on day 4 (day 3 for PHA stimulated cocultures) and quantified 18 hours later as described previously (4).

The presence of the $CD4^+D25^{+high}$ cells was associated with significant inhibition of proliferation as determined by thymidine uptake following overnight pulsing in cocultures stimulated with anti-CD3 and tetanus toxoid recall antigen (Fig. 1B). Similarly, mitogenic stimulation of $CD4^+CD25^-$ cells in the presence or absence of $CD4^+D25^{+high}$ cells at a 1:1 ratio also demonstrated significant inhibition in proliferation (Fig. 1B). The level of suppression correlated with the ratio of $CD4^+CD25^-$ : $CD4^+CD25^{+high}$ (1:1) cells in the culture, with more $CD4^+CD25^{+high}$ cells resulting in more suppression of $CD4^+CD25^-$ cell proliferation (data not shown). These results are not due to exhaustion of nutrients within the culture system because the addition of the same amount of $CD4^+CD25^-$, instead of $CD4^+CD25^{+high}$ ,cells did not cause suppression (Fig. 1B).

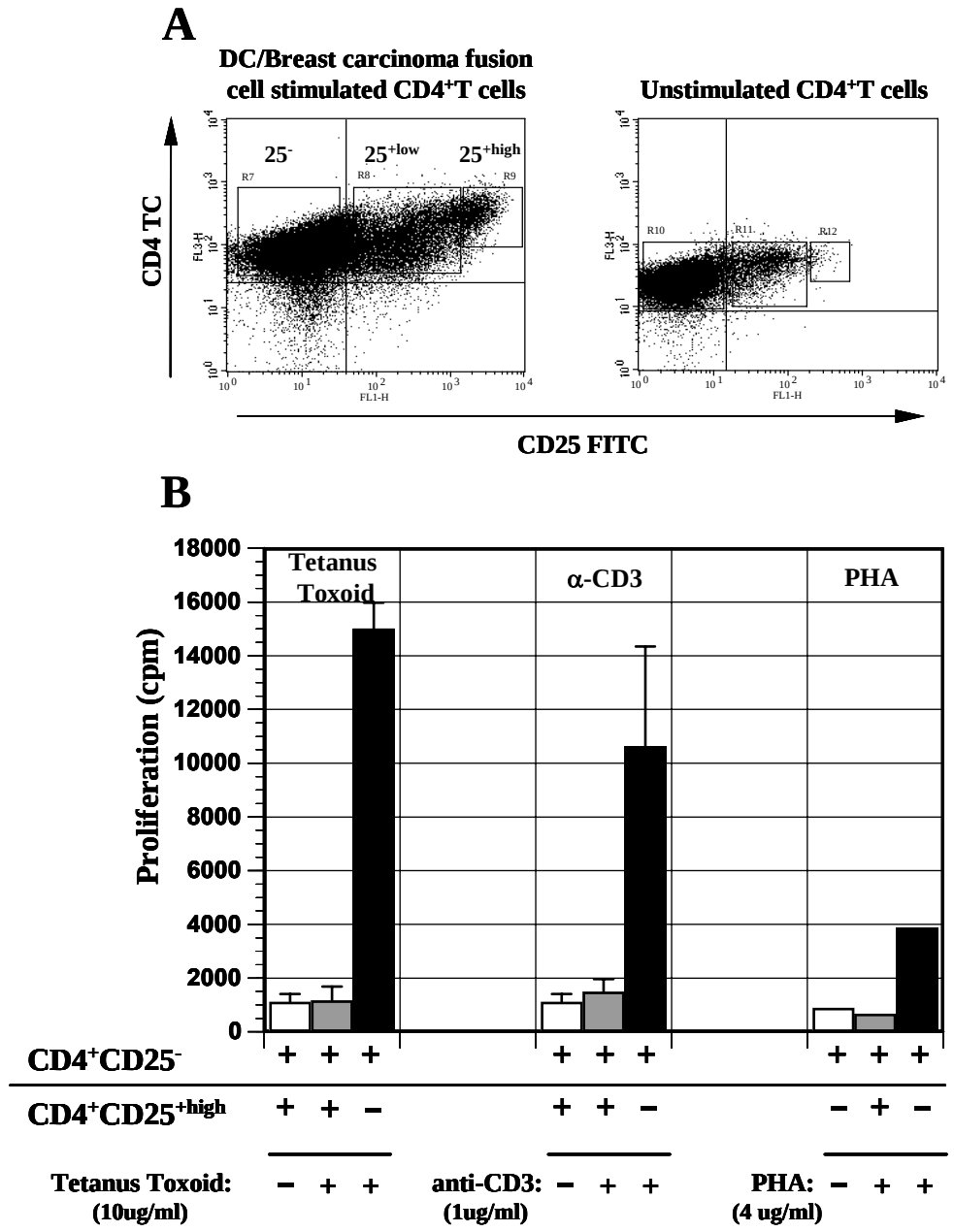



**Figure 1. Induction of $CD4^+CD25^{+high}$ regulatory T cells in the DC/breast carcinoma fusion cell cocultures**. **(A)** Representative bidimensional dot plot FACS analysis of unstimulated and DC/breast carcinoma fusion cell-stimulated T cells after positive selection of CD4 T cells followed by staining with anti-CD4 TC and anti-CD25 FITC antibodies. **(B)** Proliferation and suppression of $CD4^+CD25^-$ cells stimulated with anti-CD3 antibody, tetanus toxoid or PHA in the presence or absence of $CD4^+CD25^{+high}$ (1:1 ratio) cells. Control group of experiments consisted of addition of an equal number of $CD4^+CD25^-$ cells. The bar graph represents the mean (±SEM) of four separate experiments.

The $CD4^+D25^{+high}$ population was further analyzed for the expression of Foxp3. As shown in Fig. 2, greater than 70% of the FACS sorted $CD4^+CD25^{+high}$ T cells

expressed Foxp3, whereas the $CD4^+CD25^-$ population showed virtually no expression indicating that Foxp3 expression correlates well with the regulatory activity of the $CD4^+CD25^{+high}$ T cells (5). These data demonstrate that DC/breast carcinoma fusion cells induce the expansion of a T cell population with phenotypic and functional characteristics of regulatory T cells.

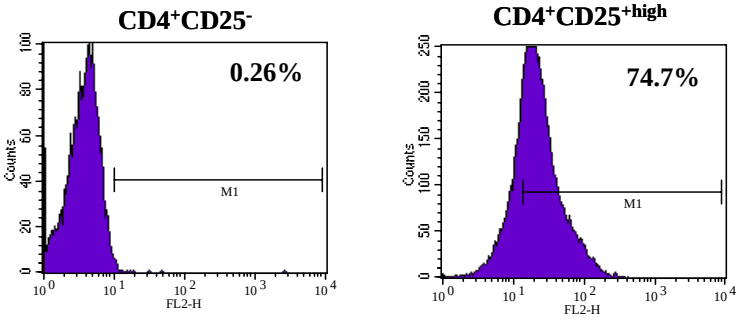
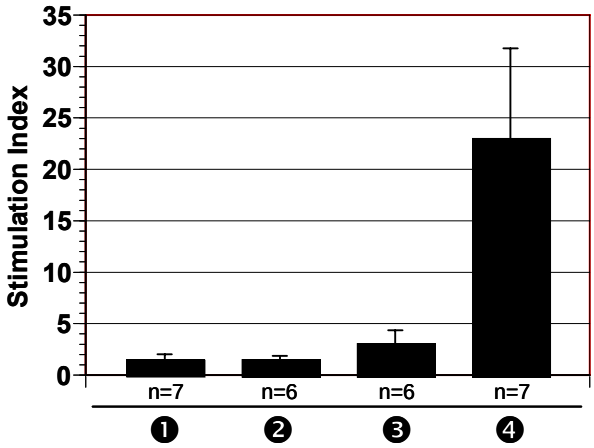



**Figure 2. Expression of Foxp3 in $CD4^+CD25^{+high}$ T cells**. FACS sorted populations of $CD4^+CD25^{+high}$ and $CD4^+CD25^-$T cells were fixed and permeabilized followed by intracellular staining with PE-conjugated Foxp3 antibody. The cells were analyzed by a single FL2-H channel. No expression of Foxp3 was observed in the $CD4^+CD25^-$ cells as compared to $CD4^+CD25^{+high}$ T cells.

## Selective expansion of activated T cells with DC/breast carcinoma fusions followed by anti-CD3/CD28

In the previous year, we examined several strategies to enhance the capacity of DC/breast carcinoma fusions to stimulate anti-tumor immunity and limit the expansion of regulatory T cells. We hypothesized that combining antigen specific stimulation with DC/tumor fusions and nonspecific ligation of the T cell costimulatory complex (CD3/CD28) would result in the activation of tumor specific lymphocytes. Indeed, in preliminary studies, we have demonstrated that combined stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 results in the expansion of tumor reactive T cells with a predominantly activated phenotype.

Over the past year, we extended these studies by examining the phenotypic and functional characteristics of T cells undergoing sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 (Fig. 3). Limited proliferation of T cells was observed following exposure to anti-CD3/CD28 alone (SI: 1.5 ±0.5 SEM; n=7) or DC/breast carcinoma fusions alone (SI 3.1 ±1.2 SEM; n=7). However, a marked increase in T cell expansion was noted when T cells were first stimulated with DC/breast carcinoma fusions and then expanded with anti-CD3/CD28 (SI: 23 ±8.73 SEM; n=7) (Fig. 3) Of note, no increase in proliferation was observed when T cells were first exposed to anti-CD3/CD28 and then cultured with DC/breast carcinoma fusions (SI: 1.6 ±0.3 SEM; n=6).




❶ ⇔ T cells stimulated with anti-CD3/CD28 alone for 2 days

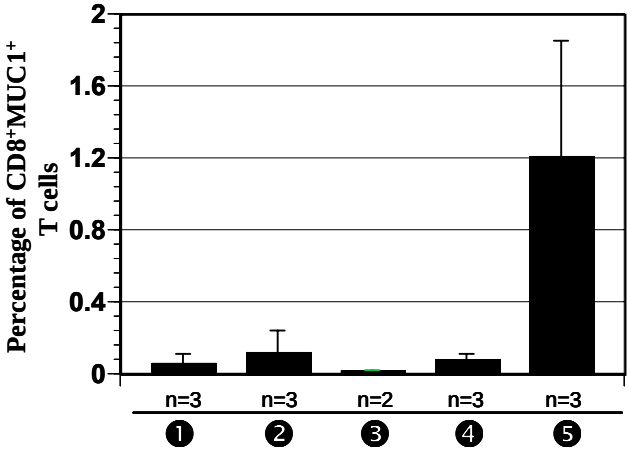
❷ ⇔T cells stimulated with anti-CD3/CD28 for 2 days and then DC/breast fusions for 5 days

❸ ⇔ T cells cocultured with DC/breast carcinoma fusion cells for 5 days

❹ ⇔ T cells cocultured with DC/breast carcinoma fusion cells for 5 days followed by stimulation with anti-CD3/CD28 for 2 days

**Figure 3. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation**. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 2 days, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared to unstimulated T cells. Mean T cell proliferation was determined for each culture condition (n=6-7). T cells were aliquoted at $1x10^5$/well in triplicate in a 96 well tissue culture plate and pulsed with 1 µCi/ml of $^3$[H]-Thymidine for a period of 18-24 h. Results were normalized by calculation of the stimulation index (SI) and are presented for each of the culture conditions.

Sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 resulted in the marked and specific expansion of tumor-reactive T cells. Importantly, exposure to anti-CD3/CD28 following fusion cell stimulation induced a 13.7 mean fold increase in MUC1 tetramer binding cells (n=3) (Fig. 4). By contrast, the percentage of MUC1 tetramer positive cells remained at baseline levels following stimulation with anti-CD3/CD28 alone.




❶ ⇔ Unstimulated cultured Tcells only

❷ ⇔ T cells stimulated with anti-CD3/CD28 alone for 2 days

❸ ⇔ T cells stimulated with anti-CD3/CD28 for 2 days and then DC/breast fusions for 5 days

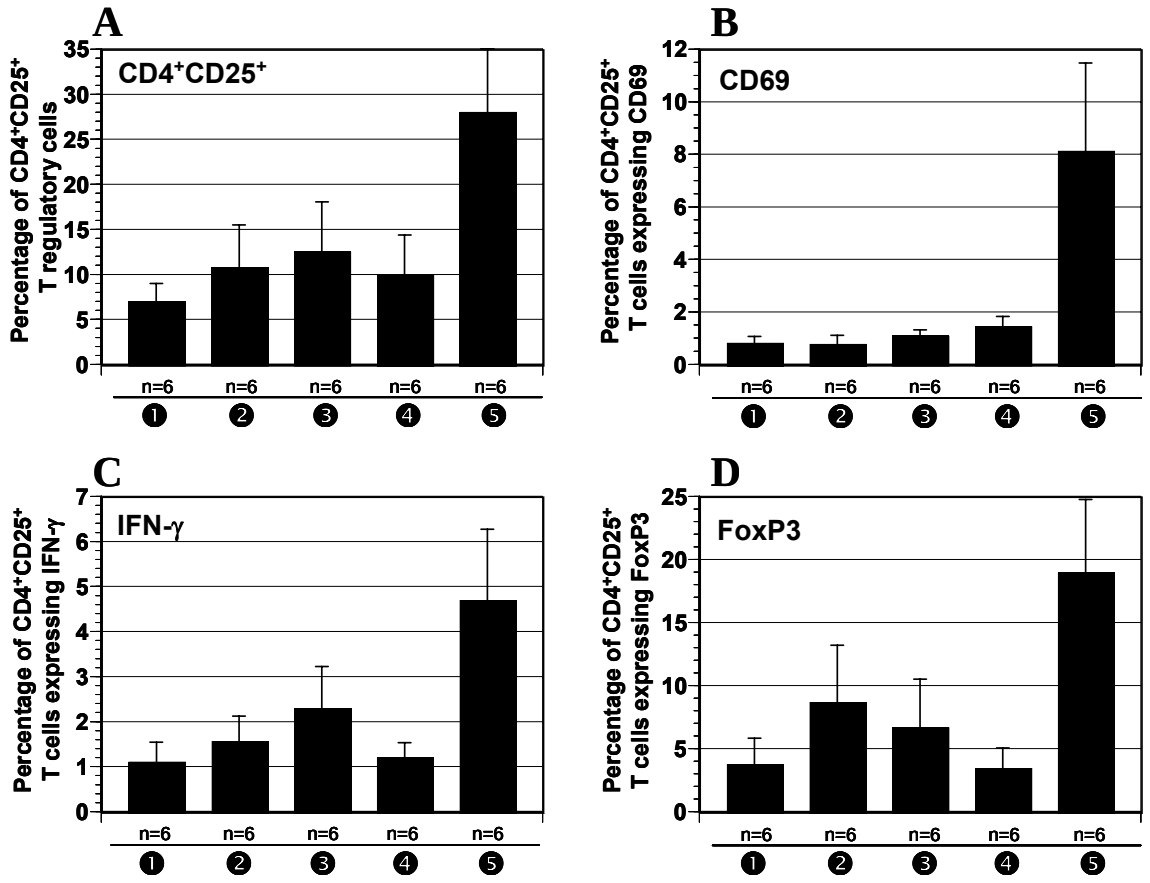
❹ ⇔ T cells cocultured with DC/breast carcinoma fusion cells for 5 days

❺ ⇔ T cells cocultured with DC/breast carcinoma fusion cells for 5 days followed by stimulation with anti-CD3/CD28 for 2 days

**Figure 4. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation**. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 2 days, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared to unstimulated T cells. Mean expression of CD8+MUC1+ T cells using PE-conjugated MUC1 specific tetramers is presented for each of the culture conditions.

With regard to the phenotype of the expanded T cell population, the percentage of T cells expressing $CD4^+CD25^+$ was markedly increased following sequential stimulation with DC/tumor fusions and anti-CD3/CD28 (28%) as compared to T cell stimulated by anti-CD3/CD28 (11%) or fusions alone (10%) (n=6) (Fig. 5A). As compared to fusion cells alone, sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 resulted in a 5 and 4 fold increase of $CD4^+CD25^+$ cells that coexpressed CD69 (Fig. 5B) and IFNγ (Fig. 5C), respectively. In contrast, an approximately 5 fold increase of regulatory T cells was also observed as manifested by an increase in $CD4^+CD25^+$ T cells that expressed Foxp3 (Fig. 5D).

These results suggest that fusion-mediated stimulation followed by anti-CD3/CD28 expansion induces increased levels of both activated and regulatory T cells.




❶ ⇔ Unstimulated cultured Tcells only

❷ ⇔ T cells stimulated with anti-CD3/CD28 alone for 2 days

❸ ⇔ T cells stimulated with anti-CD3/CD28 for 2 days and then DC/breast fusions for 5 days

❹ ⇔ T cells cocultured with DC/breast carcinoma fusion cells for 5 days

❺ ⇔ T cells cocultured with DC/breast carcinoma fusion cells for 5 days followed by stimulation with anti-CD3/CD28 for 2 days

**Figure 5. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation**. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 2 days, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared to unstimulated T cells. (**A)** Mean expression of CD4+CD25+ T cells (n=6), **(B)** CD4+CD25+CD69+ T cells (n=6), **(C)** IFNγ expressing CD4+CD25+ T cells (n=5), and **(D)** Foxp3 expressing CD4+CD25+ T cells is presented for each of the culture conditions.

## Key research accomplishments

Our pre-clinical studies have demonstrated that DC/breast carcinoma fusion cells when cocultured with T cells results in a considerable induction of Foxp3 expressing $CD4^{+}CD25^{+high}$ T cells. This population of cells were found to be potent inhibitors of $CD4^{+}CD25^{-}$ cells in a coculture system in vitro. Furthermore, our results have demonstrated that sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 results in the marked expansion of activated tumor specific T cells far in excess to that seen with either modality alone or when T cells were exposed to anti-CD3/CD28 prior to the DC/breast carcinoma fusions. Taken together, these findings suggest that DC/breast carcinoma fusions are effective antigen presenting cells, but stimulate inhibitory regulatory T cells that limit vaccine efficacy. The findings also indicate that ligation and stimulation of the TCR with anti-CD3/CD28 enhances vaccine efficacy by limiting the regulatory T cell response and promoting expansion of activated effector cells. Thus the resultant T cell population is predominantly an activated phenotype. As such, this strategy provides a promising approach to enhance vaccine efficacy and an important platform for adoptive immunotherapy with activated T cells.

## Reportable Outcomes

The results obtained from preclinical studies performed for the first 3 Tasks, together with data obtained in the past year, have been integrated into a manuscript that has been submitted for publication

## Conclusions

Our clinical protocol has received approval by the FDA, NCI/CTEP (distributor of IL-12) and Dana-Farber/Harvard Cancer Center. We have also met the requirements as outlined in the DOD review process. However, during the protracted period of DOD review, the availability of IL-12 was suspended for recertification, which has significantly delayed the initiation of the clinical trial. We have worked closely with Drs. Zweibel and Streicher at CTEP who have assumed control of the IL-12 stocks and have now completed the requisite potency testing for their release. In anticipation of trial initiation, the protocol and consent have been updated and the revised editions are currently being re-submitted to the IRB and FDA. We anticipate that the trial will be initiated in the next 3 months.

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